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(54) Title: BINDING MOLECULES CONTAINING AT LEAST AN IMMUNOGLOBULIN CONSTANT DOMAIN WITH MODIFIED ALLOTYPIC DETERMINANT

(57) Abstract

The application provides a binding molecule which has a first amino acid sequence comprising a domain with an ability to bind to a target molecule; and a second amino acid sequence substantially homologous to part or all of the constant region of a human immunoglobulin heavy chain, but which differs in an allotypic determinant; wherein the difference in the allotypic determinant results in said binding molecule having an improved effector function as compared to a binding molecule having the first amino acid sequence and part or all of the constant region of the immunoglobulin heavy chain. Also provided is a method for making a binding molecule which has a first amino acid sequence comprising a domain with an ability to bind to a target molecule and a second amino acid sequence comprising part or all of a human immunoglobulin heavy chain having an allotypic determinant of a sequence associated with a desired effector function.

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Binding molecules containing at least an immunoglobulin constant domain with modified allotypic determinant

The present invention relates to binding molecules.

Owing to their high specificity for a given antigen, antibodies and particularly monoclonal antibodies (Kohler, G. and Milstein C., 1975 Nature 256:495) represented a significant technical break-through with important consequences scientifically, commercially and therapeutically.

Monoclonal antibodies are made by establishing an immortal cell line which is derived from a single immunoglobulin producing cell secreting one form of a biologically functional antibody molecule with a particular specificity.

Owing to their specificity, the therapeutic applications of monoclonal antibodies hold great promise for the treatment of a wide range of diseases (Clinical Applications of Monoclonal Antibodies, edited by E. S. Lennox. British Medical Bulletin 1984, publishers Churchill Livingstone). Structurally, the simplest antibody (IgG) comprises four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulphide bonds (see figure 1). The light chains are of two types, either kappa or lambda. Each of the H and L chains has a region of low sequence variability, the constant region (C), differences therein giving rise to allotypes and a region of high sequence variability, the

variable region (V) differences therein giving rise to idiotypes. The antibody has a tail region (the Fc region) which comprises two or three domains of the C regions of the two H chains. The tail region is responsible for interactions with the effector systems through binding and activation of complement and Fc receptors.

The antibody also has two arms (the Fab region) each of which has a V_L and a V_H region associated with each other and each connected to a C region domain, C_L and CH_1 respectively. In addition there is a hinge region between the CH_1 and CH_2 domains. Typically the C region of an IgG H chain consists of CH_1 hinge CH_2 and CH_3. It is this pair of V regions (V_L and V_H) that differ from one antibody to another, and which together are responsible for recognising the antigen. In even more detail, each V region is made up from three complementarity determining regions (CDR) separated by four framework regions (FR). The CDRs are the most variable part of the variable regions, and they perform the critical antigen binding function. The CDR regions are derived from many potential germ line sequences via a complex process involving recombination, mutation and selection. It has been shown that the function of binding antigens can be performed by fragments of a whole antibody and this is well documented in the art.

There are a range of different immunoglobulins IgG,

IgM, IgA, IgD, IgE, known as isotypes. Of these, IgG is most commonly used therapeutically. It exists as isotypic sub-classes IgG1, IgG2, IgG3 and IgG4.

The amino acid sequence for the constant region is reasonably conserved. Nevertheless sequence variation does exist and there are 24 recognised allotypes of human immunoglobulin distributed between the different isotypes as follows:

IgG1	x 4
IgG2	x 1
IgG3	x 13
IgA2	x 2
IgE	x 1
Kappa	x 3

The allotypes represent alternative amino acid substitutions found at discrete sites in the protein sequence. These different allotypic determinants are found in different combinations within given allelic forms of genes, but not all possible combinations which theoretically might exist are in practice observed.

For example, four different allotypes of IgG1 can be seen (ie distinguished) by the immune system. These are G1m 1, 2, 3 and 17. Alternatively, combinations thereof, such as G1m (1, 17), can also be distinguished.

Antisera can be raised in other non-human species

which can see the alternative isoallotypes provided that the antibody is purified away from the other human isotypes. Such isoallotypes for which such an antisera exists have been called non-allotypes and given the designation for example, nG1m(1) which is the isoallotype of G1m(1). Thus, although a human isoallotype should not be immunogenic in humans, it can still potentially be recognized in a different species.

Of the above mentioned different allotypes of IgG1, three common allelic forms of human IgG1 occur with different frequencies within different racial groups, namely G1m (3), G1m (1, 17), and G1m (1, 2, 17) based upon their reactivities with human antisera directed against the determinants G1m 1, 2, 3 and 17.

For the purposes of clarity the sequences around the allotypic sites G1m (1) (2) and (17) are shown below for each isotype.

Site (1)

355	356	357	358	
Arg	Asp or Glu	Glu	Leu or Met	IgG1
Arg	Glu	Glu	Met	IgG2
Arg	Glu	Glu	Met	IgG3
Gln	Glu	Glu	Met	IgG4

Thus, at site (1), IgG1 may exist as several allotypes depending on whether aspartic acid or glutamic acid at

position 356, or leucine or methionine at position 358 are present.

Site 2

430	431	432	
Glu	Gly or Ala	Leu	IgG1
Glu	Ala	Leu	IgG2
Glu	Ala	Leu	IgG3
Glu	Ala	Leu	IgG4

Thus, at site (2), IgG1 may exist as either of two allotypes depending on whether glycine or alanine is present at position 431.

Site (17)/(3)

Sites (3) and (17) are alternative substitutions at the same site.

213	214	215	
Lys	Lys or Arg	Val	IgG1
Lys	Thr	Val	IgG2
Lys	Arg	Val	IgG3
Lys	Arg	Val	IgG4

Thus, at site (17)/(3), IgG1 may exist as either of two allotypes depending on whether lysine or arginine is present. The allotypes (17) and (3) cannot co-exist as they represent alternative substitutions at the same position.

Antibodies are generally raised in animals,

particularly rodents for monoclonal antibodies and therefore the immunoglobulins produced, have characteristic features specific to that species. The repeated administration of these foreign rodent proteins for therapeutic purposes to human patients can lead to harmful hypersensitivity reactions. In part, this problem has been overcome in recent years by using the techniques of recombinant DNA technology to 'humanise' non-human antibodies. Basically this involves creating a synthetic antibody having a mainly human sequence but retaining the rodent sequences determining the antibody specificity eg rodent CDRs. However problems still remain because of allotypic differences when compared with the patients own equivalent antibodies. Recently the present applicants have proposed that one way around this problem is to eliminate the allotypic variation from the constant region (see publication No. WO92/16562 corresponding to Application No. PCT/GB92/00445).

Going on from this earlier work the present applicants have now made the surprising discovery that allotypic polymorphism is to a certain extent responsible for differences in observed effector functions. Previously, differences in effector function was thought to be determined only by isotype and subclass (eg IgG1 versus IgG4) within a given isotype.

Antibodies exert their cytolytic effects via two main pathways: complement-mediated cytotoxicity (CMC) or antibody-dependent cell-mediated cytotoxicity (ADCC).

In CMC, there will be fixation of the first protein component (C1 via C1q) of the complement cascade leading to cleavage of C3, activation of the lytic pathway of the complement system, and to cytolysis of the foreign cells. In addition, the activation of C3 causes platelet aggregation, accumulation of neutrophils, and the formation of an inflammatory exudate. As a result of this, bystander tissue cells can be damaged even though they are not the immediate targets of the antibody recognition.

In ADCC, the antibody coated target cells are recognised by cytotoxic cells (eg monocytes and polymorphonuclear cells) having Fc receptors. Binding will occur between the target cells and cytotoxic cells via the antibody-Fc receptor bridge and lysis of the target cells will follow.

Different isotypes differ with regard to the effector systems with which they interact. Therefore, not all isotypes are able to activate complement equally well and Fc receptors (FcR) also show specificity of binding eg only a certain group of FcRs bind IgA, another group IgE and another group IgG. FcRs also occur as different classes and subclasses eg FcγRI, FcγRII and FcγIII for those

binding IgG (reviewed by van de Wintel and Capel 1993 Immunol. Today 14, 215-221).

Even within an antibody class, there are subclass differences in relation to effector function and these differences can be broadly stated. Thus human IgG4 does not seem to activate complement at all and binds FcγRI and FcγRII poorly. IgG1 and IgG3 are active in both complement activation and in binding to the three classes of receptor FcγRI, FcγRII and FcγRIII. IgG2 can activate complement under some circumstances (eg high antigen density) but does not bind to most FcγRs (Bruggeman et al., 1987 J. Exp. Med. 166, 1351-1361; Riechmann et al., 1988 Nature 332, 323-327; Greenwood, Clark and Waldmann 1993 Eur. J. Immunol. 23, 1098-1104).

Some receptors exist in a polymorphic form (ie as alleles) and there is an allelic form of the FcγRII receptor that can bind IgG2.

The subclasses 1-4 of IgG are very homologous in amino acid sequence (>90%), particularly if the hinge regions (which are not highly conserved) are treated separately. If the constant regions are very similar with only a few amino acid differences, yet the subclasses exhibit different effector functions, some or all of the amino acid differences may be responsible for moderating the observed functions. Experiments have been carried out in which the

residues which differ between subclasses are mutated or exchanged to discover which of them are responsible for the observed differences (reviewed in Greenwood and Clark 1993 Protein Engineering of antibody molecules for prophylactic and therapeutic applications in man (ed. Clark, M.) Publ. Academic Titles, UK (1993) p 85-100 Effector functions of matched sets of recombinant IgG subclass antibodies).

The present applicants have now made the surprising discovery that an antibody's allotype can determine its effector function. This enables the design of therapeutic molecules having a binding domain linked to a human immunoglobulin heavy chain constant region having a particularly desired effector function. Prior to the present applicants disclosure this has not been possible.

The present invention provides a binding molecule which has a first amino acid sequence comprising a domain with an ability to bind to a target molecule; and a second amino acid sequence substantially homologous to part or all of the constant region of a human immunoglobulin heavy chain, but which differs in an allotypic determinant; wherein the difference in the allotypic determinant results in said binding molecule having an improved effector function as compared to a binding molecule having the first amino acid sequence and part or all of the constant region of the immunoglobulin heavy chain.

Also provided is a method for making a binding molecule which has a first amino acid sequence comprising a domain with an ability to bind to a target molecule and a second amino acid sequence comprising part or all of a human immunoglobulin heavy chain having an allotypic determinant of a sequence associated with a desired effector function which method comprises: (a) examining one or more sequences, each comprising part or all of a human immunoglobulin heavy chain and obtaining said second amino acid sequence by either (i) selecting one of said one or more sequences on the basis of it comprising a said allotypic determinant; or (ii) by selecting one of said one or more sequences on the basis of it comprising a sequence suitable for altering to a said allotypic determinant and altering the sequence to make a said second amino acid sequence; (b) obtaining a said first amino acid sequence; (c) creating the binding molecule by either (i) chemically linking said first and second amino acid sequences; or (ii) making a nucleotide construct encoding said first and second amino acid sequences, transforming a suitable host cell with the nucleotide construct, and causing said host cell to express the binding molecule in the form of a fusion polypeptide.

The domain with an ability to bind to a target molecule may derive from any molecule with specificity for

another molecule. Thus the domain may derive from an enzyme, a hormone, a receptor (cell-bound or circulating) a cytokine or an antigen or antibody.

Of particular interest are the cytokines IL-2,IL-4, IL-5, IL-8, IL-12,IL-13 and IL-15 which are involved in the modulation of the immune response. These interleukins are referenced by Taniguchi et al (1983) Nature 302: 305 (IL-2), Yokota et al (1986) PNAS 83: 5894 (IL-4), Campbell et al (1987) PNAS 84: 6629 (IL-5), Yoshimura et al (1987) PNAS 84: 9233 (IL-8), Gubler et al (1991) PNAS 88, 4143 (IL-12), McKenzie et al (1993) PNAS 90: 3735 (IL-13), Grabstein et al (1994) Science 264: 965 (IL-15).

Also of interest are the soluble forms of the OX-40 receptor (Latza, U., et al 1994 Eur. J. Immunol. 24 : 677) and of the OX-40 ligand (Godfrey W.K., et al 1994 J. Exp. Med. 180 : 757). The human OX-40 receptor is expressed primarily on activated CD4+ T cells.

Alternatively the domain may derive from an antigen.

Alternatively the domain may be part or all of an antibody or a derivative thereof. Thus a binding molecule according to the present invention may provide a rodent originating antibody binding domain and a human immunoglobulin heavy chain having a particular allotypic determinant which predisposes the binding molecule to a particular effector function.

The above refers to an improved effector function. This means that the binding molecule may have an effector function of a different type to that of a binding molecule having the first amino acid sequence and part or all of the constant region of the immunoglobulin heavy chain. Alternatively, the effector function of the binding molecule may be of the same type as a binding molecule having the first amino acid sequence and part or all of the constant region of the immunoglobulin heavy chain, but the effector function being present to a lesser or greater degree. The improvement is essentially that the effector function is more appropriate to the particular utility of the binding molecule.

Thus in situations where it is important to maximise the binding molecules activities in complement lysis it may be appropriate to have an arginine residue at position 214 in the CH_2 domain.

Sequences for human immunoglobulin heavy chains are known and published. The sequence information can be obtained from the SwissProt and PIR databases using Lasergene software (DNAStar Limited, London UK) under accession numbers A93433, B90563, A90564, B91668, A91723 and A02146 for human Igγ-1 chain C region, A93906, A92809, A90752, A93132, A02148 for human Igγ-2 chain C region, A90933, A90249, A02150 for human Igγ-4 chain C region,

A92249, A91662, A02171 for human Igα-1 chain C region, A93828, A93829 and A02172 for human Igα-2 chain C region and A23511 for human Igγ-3 chain C region.

The effector functions associated with the various allotypes of the human immunoglobulin heavy chains may be established in accordance with the general teachings herein, as exemplified by the present applicants establishment that it is the arginine at position 214 which is necessary for lysis in the aglycosylated IgG1 antibodies and that having in the alternative a lysine or a threonine residue at this position does not give detectable lytic activity.

Then one may either select a human immunoglobulin heavy chain having the appropriate amino acid residues which predispose to a particular desired effector function or modify a human immunoglobulin heavy chain in order to make a heavy chain which has the appropriate amino acid residues which predispose to a particular effector function.

The heavy chain may then be coupled to an amino acid sequence comprising a binding domain at the protein level ie by chemical coupling methods. A variety of methods are available to the skilled person to couple antibody molecules to other moieties such as radio-nucleotides, toxins, enzymes, cytotoxic drugs and other antibody

molecules. They include covalent attachment to exposed tyrosine residues, or to the ε-amino acid side chains of aspartic and glutamic acids. Alternatively, sulphydryl groups generated by the chemical reduction of cystine residues have been used to cross-link antibody domains (Rhind, S K (1990) EP 0385601 Cross-linked antibodies and processes for their preparation). Finally, chemical modification of carbohydrate groups has been used to generate reactive groups for cross-linking purposes. These methods are standard techniques available to those skilled in the art.

Alternatively it may be more appropriate to use recombinant techniques to express the binding molecule in the form of a fusion protein. Broadly speaking this would involve making a nucleotide construct encoding the first and second amino acid sequences, using the construct to transform a suitable host cell and then causing the host cell to express the binding molecule. This may be done in accordance with standard methodologies and in general, reference may be made to "Molecular Cloning" A Laboratory Manual, eds. Sambrook, Fritsch and Maniatis, Cold Spring Harbor Laboratory Press 1989.

Thus, the domain may comprise an antibody binding domain. The antibody binding domain may be non-human. The antibody binding domain may be of rodent origin.

The domain may comprise part or all of a cytokine. The cytokine may be selected from IL-2, IL-4, IL-5, IL-8, IL-12, IL-13 and IL-15. In particular the cytokine may be IL-12.

The domain may comprise part or all of a soluble form of a cell bound ligand or a cell bound receptor. The cell bound receptor may comprise the OX-40 receptor. The cell bound ligand may comprise the OX-40 ligand.

The human immunoglobulin heavy chain may be of the isotype IgG1. The difference in an allotypic determinant may be in the CH_1 domain. The difference in an allotypic determinant may comprise the presence or absence of arginine at position 214. Where arginine is absent from position 214, lysine or threonine may instead be present. The difference in an allotypic determinant may be in the CH_2 domain. The difference in an allotypic determinant may comprise the presence or absence of asparagine at position 297. Where asparagine is absent from position 297, alanine may instead be present.

The second amino acid sequence may differ from the part or all of the constant region of an immunoglobulin heavy chain in more than one allotypic determinant. The differences may be in both the CH_1 and CH_2 domains. An amino acid other than arginine (eg lysine or threonine) may be present at position 214 of the CH_1 domain and an amino

acid other than asparagine (eg alanine) may be present at position 297 of the CH_2 domain.

The present invention also provides a pharmaceutical preparation which comprises a binding molecule as above.

The present invention also provides a reagent which comprises a binding molecule as above.

The present invention also provides a method of treating a patient which comprises administering a pharmaceutical as above.

Also provided is use of a binding molecule as above in the preparation of a pharmaceutical to modify an immune response.

Also provided is a packet comprising a pharmaceutical or reagent as provided above along with instructions specifying use thereof.

The present invention also provides a recombinant construct which comprises a nucleotide sequence coding for a binding molecule as above. Also provided are cloning and expression vectors which comprise a recombinant construct as above, along with host cells comprising such vectors and transgenic animals carrying such recombinant constructs.

In order that the present invention is more fully understood, embodiments will be described in more detail by way of example only and not by way of limitation with

reference to the figures listed below.

Figure 1 illustrates the structure of an IgG antibody (as discussed earlier).

Figure 2 shows the M13TG131 cloning vector containing the human gamma-1 constant region and showing cloning sites and modified polylinker.

Figures 3 shows a complement dependent assay for the killing of autologous human peripheral blood lymphocytes with five CAMPATH-1 (CDw52) specific antibody constructs, TF57-19, MTF121, MTF123, MTF133 and MTF134. The degree of lysis was calculated from the % specific release of the radioisotope ^{51}Cr. The results for the three allotypes of human γ1 agreed with earlier findings, ie there is no significant difference between TF57-19, MTF121 and MTF123 when assayed under conditions for optimal lysis. Aglycosylated mutants of TF57-19 and MTF121 were constructed by mutating the Asn 297->Ala giving rise to MTF133 and MTF134 respectively. As expected, these new constructs had a reduced activity in this assay, but unexpectedly there was a significant difference between them, as MTF134 still gave some residual lysis. As these two constructs differ only by allotypic residues in the CH_1 and CH_3 domains, being identical in the hinge and CH_2 regions, this is an interesting observation.

Figure 4 shows results of an experiment following on

from the results given in Figure 3. A new construct BTF186 was prepared and compared with the previous constructs. Note that the results are expressed as ^{51}Cr released in cpm and have not been converted to % specific release. The results for the 5 original constructs agree very well with the results in Figure 3 and show the reproducibility in the assay. Quite clearly, construct BTF186 is more closely related to MTF134, than to MTF133, in terms of the lytic function. Referring to Table 1 it can be seen that this ability to give lysis correlates with the Arg 214 in the CH_1 domain and is independent of the residues found at position 356 and 358 in the CH_3 domain.

Figure 5 shows the results of an experiment following on from the observations in Figure 4. The results shown, confirms that it is the Arg at position 214 which is necessary for the observed lysis in the aglycosylated IgG1 antibodies and that having the alternative residues of a Lys or a Thr at this position does not give detectable lytic activity.

The methods used herein are as described in WO92/16562.

The data disclosed herein is based on the use of different mutated γ1 heavy chain constant regions expressed along with the heavy chain V-region and the light chain for the CAMPATH-1 specificity (against antigen CDw52). The

vectors M13TG131 containing the γ1 gene (see Fig. 2), the expression vector pSVgpt containing the V_H gene, methods of mutation and methods of expression are all fully described in WO92/16562.

The original human IgG1 "wild type" construct, Y0 transfectant, clone TF57-19 was of immunoglobulin allotype Glm(1,17). An alternative allele was constructed by mutating the residues in CH_1 and CH_3 (using oligonucleotide primers MO1 and MO2 as described in WO92/1652) to give the allotype Glm(3), clone MTF121. In a second step, the Glm(3) allotype was eliminated by replacing the Arg 214 with a Thr (using oligonucleotide primers MO2 and MO4 as described in WO92/16562) to give the "null" allotype of clone MTF123. Comparison of these constructs under optimal conditions indicates that they were very similar in their abilities to mediate autologous human complement dependent lysis of human lymphocytes.

For these new experiments, some modifications to the above described constructs have been made. The major and starting modification is a further mutation in the CH_2 domain encoding intron to alter the conserved site of N-linked glycosylation by mutating the Asn 297 to Ala. This was achieved by site directed mutagenesis (as used previously for the allotype mutants) of the appropriate sequence using an oligonucleotide primer "MO3" with the

following sequence.

5' CCG GTA CGT GCT AGC GTA CTG CTC CTC 3'

to give the final γ1 sequence of CH_2 of

297

Glu Glu Gln Tyr Ala Ser Thr Tyr Arg

5' GAG GAG CAG TAC GCT AGC ACG TAC CGG 3'

3' CTC CTC GTC ATG CGA TCG TGC ATG GCC 5'

The wild type γ1 Glm(1,17) sequence was mutated in the M13TG131 vector with oligonucleotide MO3. The sequence was checked and the constant region gene was recloned into the pSVpgt vector containing the CAMPATH-1 VH region and transfected into YO cells along with the light chain to give the expressed clone MTF133. In a similar way, the allele Glm(3) was mutated and expressed as clone MTF134 (see Table 1 for summary).

Supernatant from cultures of these two clones were firstly screened for production of human IgG1 antibody with CAMPATH-1H specificity using the Enzyme-Linked Immuno-Sorbent Assay (ELISA) method described in Example 1 of WO92/01652. Positive clones were grown up to bulk cultures and the antibody concentrated by precipitation with 50% saturated ammonium sulphate. Following dialysis into phosphate-buffered saline (PBS), the concentration of the antibodies were estimated by a quantitative ELISA. Complement dependent lysis was carried out as described in

Example 2 of WO92/16562. Human peripheral blood mononuclear cells were labelled with ^{51}Cr, the cells were added to titrations of the antibodies and human serum was added as a source of complement. The percentage of specific ^{51}Cr release was calculated by measuring released cpm after a 1 hour incubation at 37°C. From the literature and their own studies with an aglycosyl γ1 antibody to human CD3 [Bolt, S., et al (1993) Eur. J. Immunol. 23, p 403-411 The generation of a humanised, non-mitogenic CD3 monoclonal antibody which retains in vitro immunosuppressive properties] and another to mouse CD8 [Isaacs, J. D., et al (1992) Immunol. 148, p 3062-3071 Therapy with monoclonal antibodies - an in vivo model for the assessment of therapeutic potential], the present applicants had fully expected the aglycosyl mutants to be equally defective in complement mediated lysis compared to the wild type glycosylated γ1 antibody. To their surprise they found that unlike the Glm(1,17) aglycosyl antibody MTF133 the Glm(3) aglycosyl antibody MTF134 had some residual lytic ability (see Figure 3). The relevant amino acid residues for the two antibodies MTF133 and MTF134 are shown below.

Name	Allotype	Phenotype	Amino Acids					
			214	297	355	356	357	358

MTF133	G1m(1,17)	Aglycosylated	Lys Ala Arg Asp Glu Leu
MTF134	G1m(3)	Aglycosylated	Arg Ala Arg Glu Glu Met

As these two antibodies differ from each other only in the Arg/Lys at 214 in the CH_1 domain and the Glu/ASP at 356 and in the Met/Leu at 358 in the CH_3 domain, it became of interest to resolve what was the crucial difference responsible for the observations, particularly because all previous studies have tended to indicate that it is the CH_2 domain which is crucially important for complement dependent lysis [eg Greenwood, J.D., et al (1993) Eur. J. Immunol. 23 p 1098-1104 Structural motifs involved in human IgG antibody effector functions and Greenwood, J.D., and Clark. M.R., in: Protein engineering of antibody molecules for prophylactic and therapeutic applications in man (ed. Clark M.R.) Pub. Academic Titles, UK (in press 1993) Effector functions of matched sets of recombinant IgG subclass antibodies] and the above result indicates a possible role for the CH_1 and/or CH_3 sequences.

To resolve this, the present applicants prepared some more antibody constructs. Using a PstI restriction fragment from the M13TG131 γ1 containing vectors, it is possible to remove the CH_1 encoding exon complete from one construct and to swap it with the homologous fragment from another (see Figure 2). In this way, the CH_1 domains were

swapped and expressed clones were made in which Arg 214 was present with Asp 356, Leu 358 (clone BTF186) and another in which Lys 214 was present with Glu 356, Met 358 (clone BTF187) (see summary in Table 1). A third CH_1 swap mutant was constructed in which the "null" allotype Thr 214 was present with Asp 356, Leu 358 (clone BTF188). The relevant amino acid residues for the three new antibody constructs BTF186, BTF187 and BTF188 are shown below.

Name	Allotype	Phenotype	Amino Acids					
			214	297	355	356	357	358
BTF186	G1m(1,3)	Aglycosylated	Arg	Ala	Arg	Asp	Glu	Leu
BTF187	G1m(17)	Aglycosylated	Lys	Ala	Arg	Glu	Glu	Met
BTF188	Null at 214	Aglycosylated	Thr	Ala	Arg	Asp	Glu	Leu

All three of these new constructs were recloned into pSVgpt, expressed and tested by ELISA and by complement assay as above. The results (see Figures 4 and 5) indicate quite clearly that it is the Arg 214 difference in the CH_1 which correlates with the observed functional difference. The applicants have found that the results are highly reproducible and are consistent with three different donors of the cells and complement being used.

Enzyme linked immunoadsorbent assays were used to allotype the secreted antibodies generated by the genetic

constructs and these confirm the presence of the expected allotypes (including the revised data for BTF188). The assays were adapted from the methods described in Cobbold, S.P., et al., Meth. Enzymol. 1990 127, p 19-24 and were carried out as described briefly below.

Monoclonal antibodies specific for the three Glm allotypes 1, 3 and 17 (also known as a, f and z respectively) were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusions Service (Plesmanlaan 125, 1066 CX Amsterdam)

Code	Specificity	Clone	Batch
R2102	anti-Glm(a)	5E7	MG102-P1
R2104	anti-Glm(f)	5F10	MG104-P1
R2101	anti-Glm(z)	5A1	MG101-P1

These were used to coat the surface of the wells of three microtitre plates using a volume of 50µl/well and a protein concentration of 10µg/ml. The plates were then washed with phosphate buffered saline (PBS) containing 0.1% w/v of Bovine Serum Albumen (BSA) and were then blocked with PBS 1% BSA. The antibodies to be tested were resuspended in PBS to approximately 10-100µg/ml and were dispensed onto the three different coated plates in duplicate wells. After 1 hours incubation, the plates were again washed with PBS 0.1% BSA and a biotinylated

monoclonal antibody (NH3/41.34) specific for human κ light chains was added at a concentration of 10μg/ml as a detection reagent. Bound biotinylated antibody was detected with subsequent steps of streptavidin peroxidase followed by the substrate o-phenylenediamine in citrate-phosphate buffer. The conversion of substrate was determined by measuring the absorbance at 495nm. Results from the three assays were combined in Table 2.

The functional differences identified as existing by the present applicants are likely to be of importance depending upon the therapeutic use of a given antibody. Firstly it seems reasonable to assume that the trends in the functional differences seen here would also be true for other specificities than just CAMPATH-1H, although the effects may be of a different magnitude in plateau and titre. Secondly it is also likely that although it was necessary to make the aglycosyl mutants to observe these differences with CAMPATH-1 specific antibodies, the functional differences might still be there, but less easy to see under optimal conditions with glycosylated antibodies (CAMPATH-1 is noted as an exceptionally good target for complement dependent lysis). Thus in situations where it is important to have an antibody with the best activity in complement lysis it may be appropriate to use the Arg 214 variants as in allotype Glm(3). Alternatively

in situations where no effector functions are required, as in the example of the non-mitogenic CD3 antibody, then a mutant with Lys or Thr at 214 combined with a conversion of asparagine at position 297 to alanine or some other amino acid would be appropriate.

These results indicate a role for the CH_1 in activation of complement and also point at possible functional differences in immunoglobulin alleles which might become apparent under some experimental conditions.

From all the above it can be seen that some of the residues which differ between two alleles of the same subclass are in some cases identical with the residues found in a similar position in a different subclass.

For example in the CH_1 at position 214, arginine is found in IgG4 and IgG3 whilst threonine is found in IgG2. In IgG1 the amino acid at position 214 depends on the allotype, such that arginine is found in the allotype (G1m(3) and lysine is found in the allotype G1m(17). Thus it follows that in this region the sequence of the allotype G1M(3) is more similar to IgG3 and IgG4 than is the allotype G1m(17). In the work reported herein, the applicants have shown that the IgG1 antibody with an arginine at position 214 (G1m(3)) is more active in complement activation that the IgG1 antibody with a lysine at position 214 (G1m(17)). Putting this observation

together with the homology between subclasses, it seems reasonable to propose that the arginine at position 214 in IgG3 may play some important role in moderating the function of that other subclass. [This however does not preclude the possibility that some additional amino acid changes at a different site may serve to counteract the effect for example the changes in the CH_2 domain of IgG4 which completely abolish complement activation]. In support of this is the observation that an aglycosyl IgG3 antibody (Lund et al 1990 Mol. Immunol. 27, 1145-1153) has some residual activity in complement activation. It seems reasonable therefore, to identify the arginine at 214 as a likely candidate for causing the observed difference between aglycosyl IgG3 and aglycosyl IgG1 G1m(17). Similar arguments can be extended to other allotypes of the four subclasses in which the residues present in one allotype of one subclass are identical to residues found at that position in another subclass and may therefore contribute to the observed functional differences between subclasses. To test this, the skilled person could easily extend the approach described here for IgG1 allotypes to other subclasses ie make different mutants in which only some of the allelic residues are altered at any one time and see if any of these correlate with functional changes between mutants.

Thus it follows from the above, that one can moderate the functions of one subclass by introducing mutations at positions homologous to given alleles of another subclass, ie if the arginine at position 214 in IgG3 were changed to a lysine (found in G1m(17) but not usually in IgG3), then the activity in complement activation of the mutant IgG3 should be reduced compared to wild type IgG3.

Another important point illustrated by the work described herein is the identification of residues in the CH_1 which seem to affect complement activation. Previous work has always tended to identify the much stronger effects due to differences in the CH_2 domain. Thus there is functional significance to allotypic residues in all parts of the antibody not just the CH_2.

The foregoing teaches that allotypic polymorphism is to a certain extent responsible for different effector functions. Thus it allows the creation of binding molecules having a heavy chain which predisposes the molecule to a particular effector function.

It will be possible to target immune effector mechanisms to various cell types by attaching different molecules to the N terminal of an IgFc fragment (comprising a pair of second amino acid sequences each comprising part or all of a human immunoglobulin heavy chain having an allotypic determinant of a sequence associated with a

desired effector function) and using the specificity of the attached molecule to label particular cells for destruction. For instance, attachment of a soluble form of a transmembrane receptor, coding for the extracellular portion of that receptor, to the IgFc would produce a molecule capable of binding to a cell expressing a ligand for the receptor (eg binding of soluble CD40.Fc to cells expressing the CD40 ligand, Armitage et al (1992), Nature 347: 80-82).

Alternatively, one could attach a cytokine to the IgFc fragment and use that hybrid molecule to label cells expressing the receptor for the cytokine (eg binding of IL-2/IgG1 chimeric molecule to cells expressing IL-2 receptors, Landolfi (1991), J. Immunol. 146: 915-919). This attachment to IgFc may be by chemical linkage or by recombinant DNA technology to produce a fusion protein.

Cells thus labelled could then be destroyed via immune effector activation directed by the IgFc portion of the fusion protein.

Table 1 Summary of expressed clones and their characteristics

Clone Name	M13 Construct	Constant Region	Phenotype	Allotype	Position 214	297	356	358	Oligos Used
TF57-19	G1 w.t.	γ1 wild type	fully wild type	Glm(1,17)	Lys	Asn	Asp	Leu	None
MTF121	G1M1M2	γ1 mutant	γ1 allele	Glm(3)	Arg	Asn	Glu	Met	M01.M02
MTF123	G1M2M4	γ1 mutant	γ1 "null" allele	"null"	Thr	Asn	Glu	Met	M02.M04
MTF133	G1M3.	γ1 mutant	aglycosylated w.t.	Glm(1,17)	Lys	Ala	Asp	Leu	M03
MTF134	G1M1M2M3	γ1 mutant	aglycosylated allele	Glm(3)	Arg	Ala	Glu	Met	M01.M02.M03
BTF186	G1M1M3.	γ1 mutant	aglycosylated allele	Glm(1,3)	Arg	Ala	Asp	Leu	M01.M03
BTF187	G1M2M3	γ1 mutant	aglycosylated allele	Glm(17)	Lys	Ala	Glu	Met	M02.M03
BTF188	G1M3M4	γ1 mutant	aglycosylated "null"	"null",Glm(1)	Thr	Ala	Asp	Met	M03.M04

"Clone Name" gives the name given to the final expressed product in Y0 cells transfected with a pSVgptV_HCAMPATH construct. The "M13 Construct" is the name of the intermediate M13TG131 construct used in derivation of the final antibody. Also given is information on the expressed constant region phenotype and the allotype of the antibodies together with the amino acids found at the relevant positions in the γ1 sequence. The final column gives details of the mutagenic oligonucleotides used in for the derivation of the construct.

Table 2 Enzyme linked immunoadsorbent assay to detect immunoglobulin allotypes of transfectants

Table 2a

		MTF121	MTF123	MTF133	MTF134	BTF186	BTF187	BTF188	TF57-19	CNTRL
Anti G1m(a)	Anti G1m(1)	0.33	0.39	1.08	0.31	0.89	0.61	0.99	1.55	0.13
Anti G1m(f)	Anti G1M(3)	0.82	0.39	0.31	0.95	0.82	0.49	0.34	0.31	0.31
Anti G1m(z)	Anti G1m(17)	0.63	0.83	1.61	0.56	0.72	1.55	0.52	1.89	0.21

Table 2b

		MTF121	MTF123	MTF133	MTF134	BTF186	BTF187	BTF188	TF57-19	CNTRL
Anti G1m(a)	Anti G1m(1)	0.14	0.18	0.67	0.13	0.53	0.33	0.61	1.00	0.00
Anti G1m(f)	Anti G1M(3)	0.80	0.12	0.01	1.00	0.80	0.28	0.05	0.00	0.00
Anti G1m(z)	Anti G1m(17)	0.25	0.37	0.83	0.21	0.30	0.80	0.19	1.00	0.00

Table 2c

		MTF121	MTF123	MTF133	MTF134	BTF186	BTF187	BTF188	TF57-19	CNTRL
Anti G1m(a)	Anti G1m(1)	-	-	+	-	+	-	+	+	
Anti G1m(f)	Anti G1M(3)	+	-	-	+	+	-	-	-	
Anti G1m(z)	Anti G1m(17)	-	-	+	-	-	+	-	+	

The results of three ELISA assays to detect the allotypes G1m(1), G1m(3) and G1m(17) are summarised above. In 2a the results are given as the average of duplicate wells in terms of the measured absorbance at 495nm. For convenience of comparison between the three assays these results are then normalised in table 2b to a scale of 0 (lowest value) to 1 (highest value). Finally in 2c the results are interpreted in terms of a positive result being greater than 0.5 and a negative less than 0.5. The results confirm the expected allotypes predicted from the genetic constructs detailed in Table 1. The allotypes are also given in terms of the two recognised systems of notation ie in both their numeric and the equivalent alphabetical numenclature a=1, f=3, z=17.

CLAIMS

1. A binding molecule which has

a first amino acid sequence comprising a domain with an ability to bind to a target molecule; and

a second amino acid sequence substantially homologous to part or all of the constant region of a human immunoglobulin heavy chain, but which differs in an allotypic determinant

wherein the difference in the allotypic determinant results in said binding molecule having an improved effector function as compared to a binding molecule having the first amino acid sequence and part or all of the constant region of the immunoglobulin heavy chain.

2. A binding molecule according to claim 1 wherein the domain comprises an antibody binding domain.

3. A binding molecule according to claim 2 wherein the antibody binding domain is non-human.

4. A binding molecule according to claim 3 wherein the antibody binding domain is of rodent origin.

5. A binding molecule according to claim 1 wherein the domain comprises part or all of a cytokine.

6. A binding molecule according to claim 5 wherein the

cytokine comprises anyone of IL-2, IL-4, IL-5, IL-8, IL-12 IL-13 and IL-15.

7. A binding molecule according to claim 6 wherein cytokine comprises IL-12.

8. A binding molecule according to claim 1 wherein the domain comprises part or all of a soluble form of a cell bound ligand or a cell bound receptor.

9. A binding molecule according to claim 8 wherein the cell bound receptor comprises the OX-40 receptor.

10. A binding molecule according to claim 8 wherein the cell bound ligand comprises the OX-40 ligand.

11. A binding molecule according to any one of claims 1 to 10 wherein the human immunoglobulin heavy chain is of the isotype IgG1.

12. A binding molecule according to any one of claims 1 to 11 wherein the difference in an allotypic determinant is in the CH_1 domain.

13. A binding molecule according to any one of claims 1 to 12 wherein the difference in an allotypic determinant comprises the presence or absence of arginine at position 214.

14. A binding molecule according to any one of claims 1 to 11 wherein the difference in an allotypic determinant is in the CH_2 domain.

15. A binding molecule according to claim 14 wherein the difference in an allotypic determinant comprises the presence or absence of asparagine at position 297.

16. A binding molecule according to any one of claims 1 to 15 wherein the second amino acid sequence differs from the part or all of the constant region of an immunoglobulin heavy chain in more than one allotypic determinant.

17. A binding molecule according to claim 16 wherein the differences are in both the CH_1 and CH_2 domains.

18. A binding molecule according to claim 17 which comprises an amino acid other than arginine at position 214 of the CH_1 domain and an amino acid other than asparagine at position 297 of the CH_2 domain.

19. A pharmaceutical preparation which comprises a binding molecule according to any one of claims 1 to 18.

20. A reagent which comprises a binding molecule according to any one of claims 1 to 18.

21. A recombinant construct which comprises a nucleotide

sequence coding for a binding molecule according to any one of claims 1 to 18.

22. A cloning or expression vector which comprises a construct according to claim 21.

23. A host cell which comprises a vector according to claim 22.

24. A transgenic animal which carries a recombinant construct according to claim 21.

25. A method for making a binding molecule which has a first amino acid sequence comprising a domain with an ability to bind to a target molecule and a second amino acid sequence comprising part or all of a human immunoglobulin heavy chain having an allotypic determinant of a sequence associated with a desired effector function which method comprises:

(a) examining one or more sequences, each comprising part or all of a human immunoglobulin heavy chain and obtaining said second amino acid sequence by either (i) selecting one of said one or more sequences on the basis of it comprising a said allotypic determinant; or (ii) by selecting one of said one or more sequences on the basis of it comprising a sequence suitable for altering to a said allotypic determinant and altering the sequence to make a said second amino acid sequence;

(b) obtaining a said first amino acid sequence;

(c) creating the binding molecule by either (i) chemically linking said first and second amino acid sequences; or (ii) making a nucleotide construct encoding said first and second amino acid sequences, transforming a suitable host cell with the nucleotide construct, and causing said host cell to express the binding molecule in the form of a fusion polypeptide.

26. A method according to claim 25 wherein the domain is an antibody binding domain.

27. A method according to claim 26 wherein the antibody binding domain is non-human.

28. A method according to claim 27 wherein the antibody binding domain is of rodent origin.

29. A method according to claim 25 wherein the domain comprises part or all of a cytokine.

30. A method according to claim 29 wherein the cytokine comprises any one of IL-2, IL-4, IL-5, IL-8, IL-12, IL-13 and IL-15.

31. A method according to claim 30 wherein the cytokine comprises IL-12.

32. A method according to claim 25 wherein the domain comprises part or all of a soluble form of a cell bound ligand or a cell bound receptor.

33. A method according to claim 32 wherein the cell bound receptor comprises the OX-40 receptor.

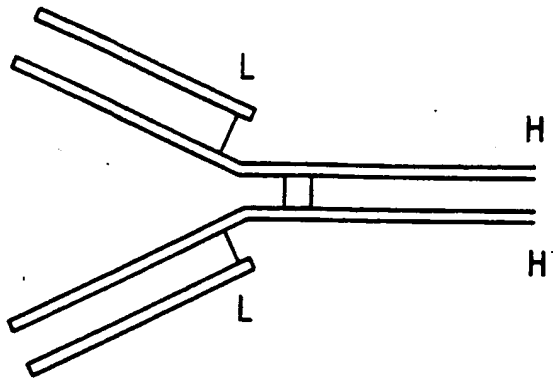
34. A method according to claim 32 wherein the cell bound ligand comprises the OX-40 ligand.

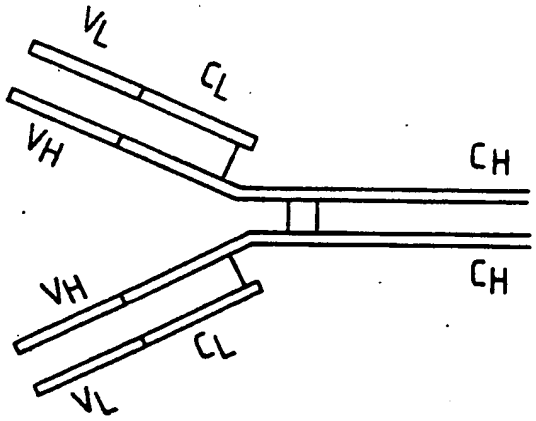
35. A method according to claim 25 wherein human immunoglobulin heavy chain is of the isotype IgG1.

36. A method according to any one of claims 25 to 35 wherein the binding molecule comprises an amino acid other than arginine at position 214 of the CH_1 domain of the human immunoglobulin heavy chain and/or an amino acid other than asparagine at position 297 of the CH_2 domain of the immunoglobulin heavy chain.

38. A method of treating a patient which comprises administering a pharmaceutical according to claim 19.

Fig.1.





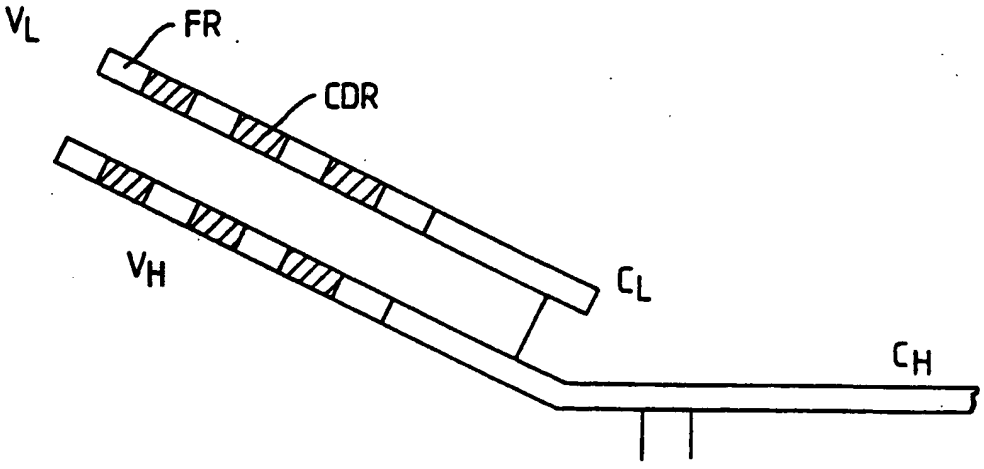
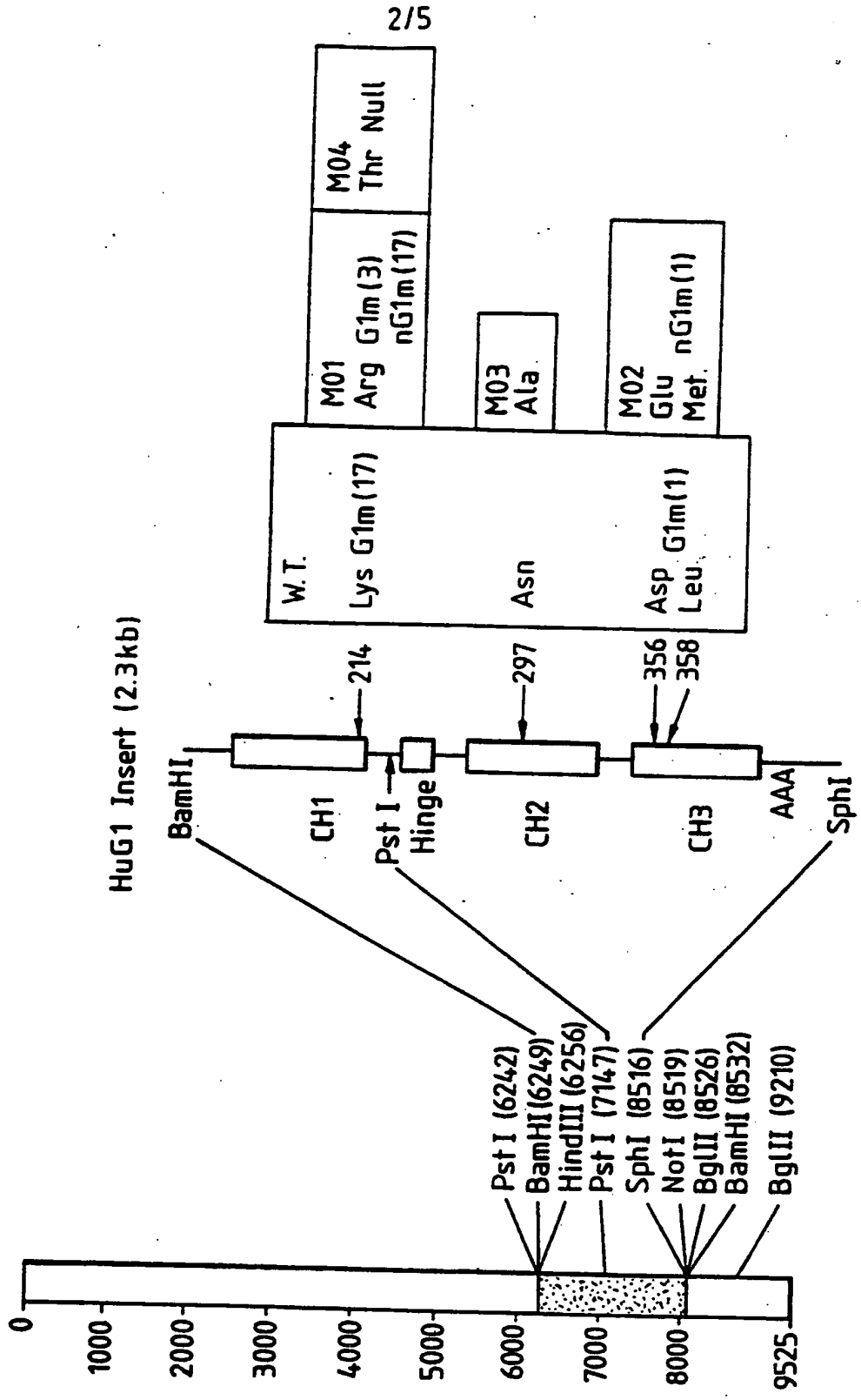


Fig.2.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M03 Ala
M02 Glu Met nG1m(1)
W.T. Lys G1m(17) Asn Asp G1m(1) Leu
214
297
356
358
HuG1 Insert (2.3kb)
BamHI
CH1
Pst I
Hinge
CH2
CH3
AAA
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BamHI (6249)
HindIII (6256)
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SphI (8516)
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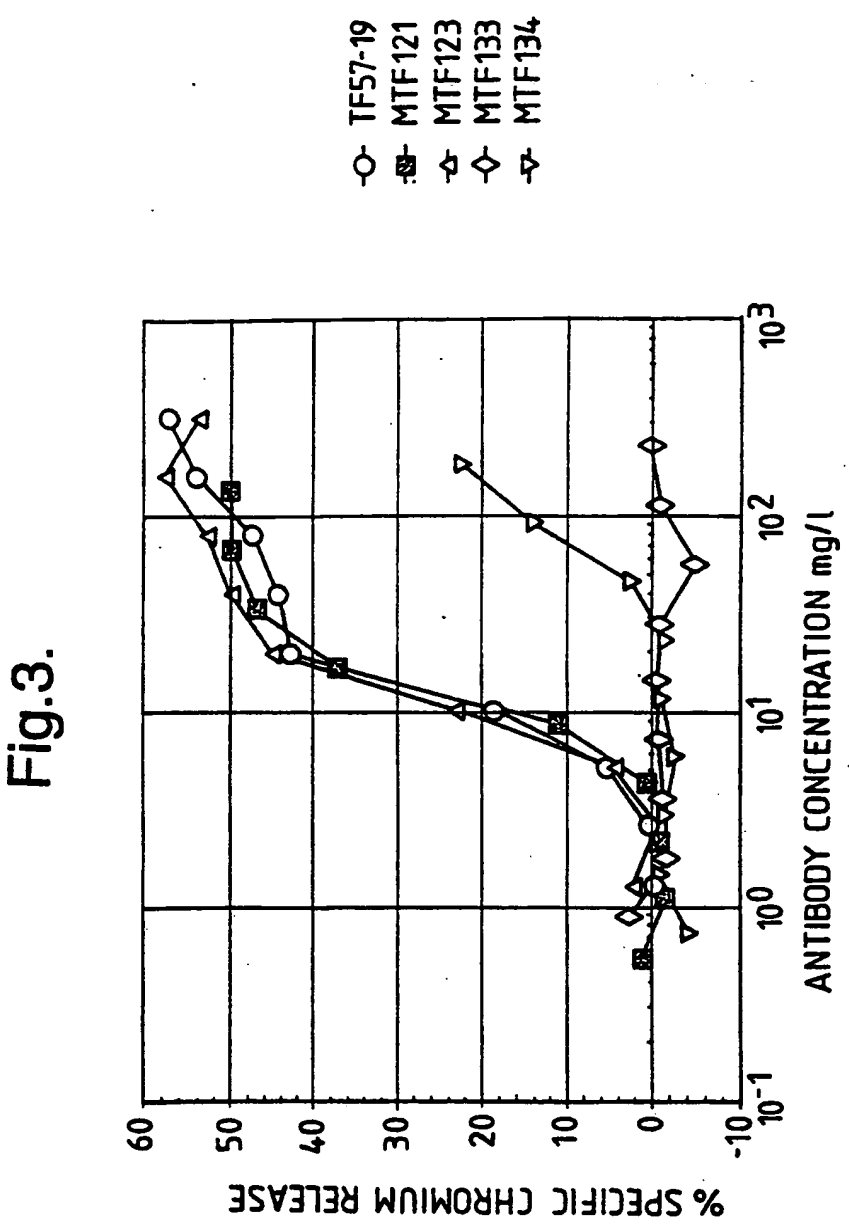
Fig.3.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MTF123
MTF133
MTF134
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40
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ANTIBODY CONCENTRATION mg/l
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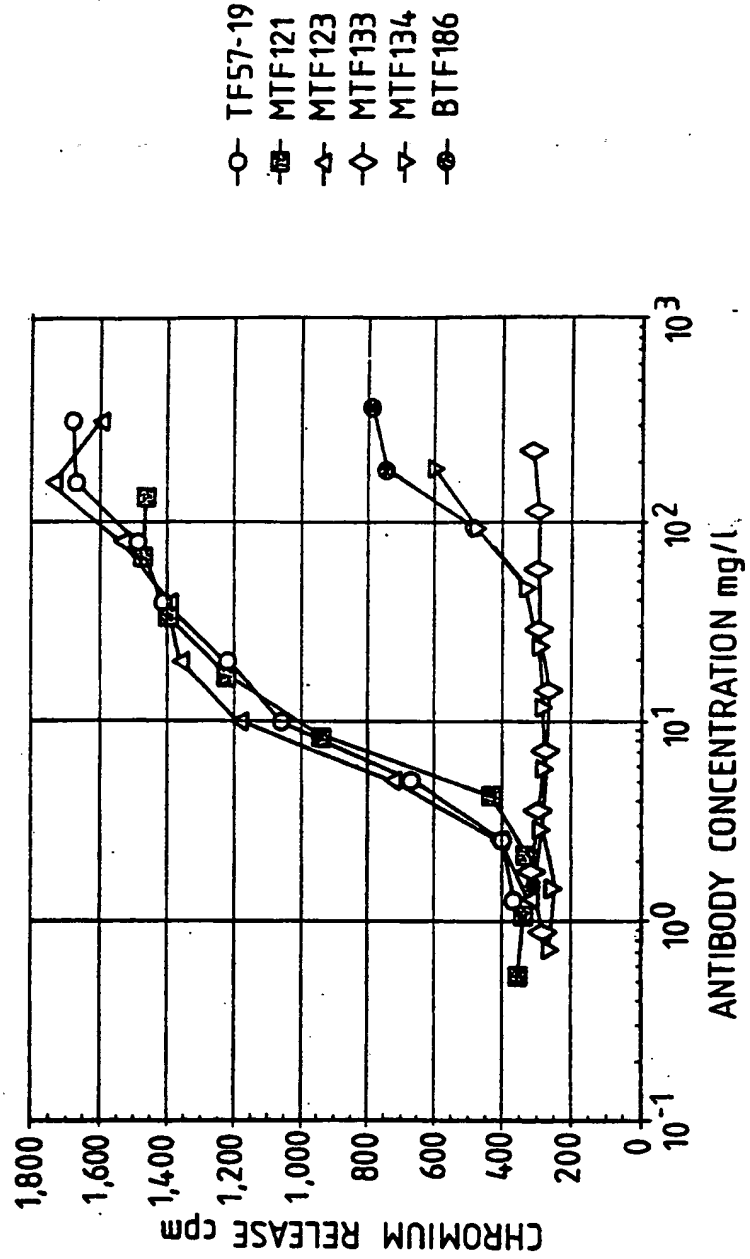
10^2
10^1
10^0
10^{-1}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MTF123
MTF133
MTF134
BTF186
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1,200
1,000
800
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ANTIBODY CONCENTRATION mg/l
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10^{1}
10^{2}
10^{3}

Fig.4.

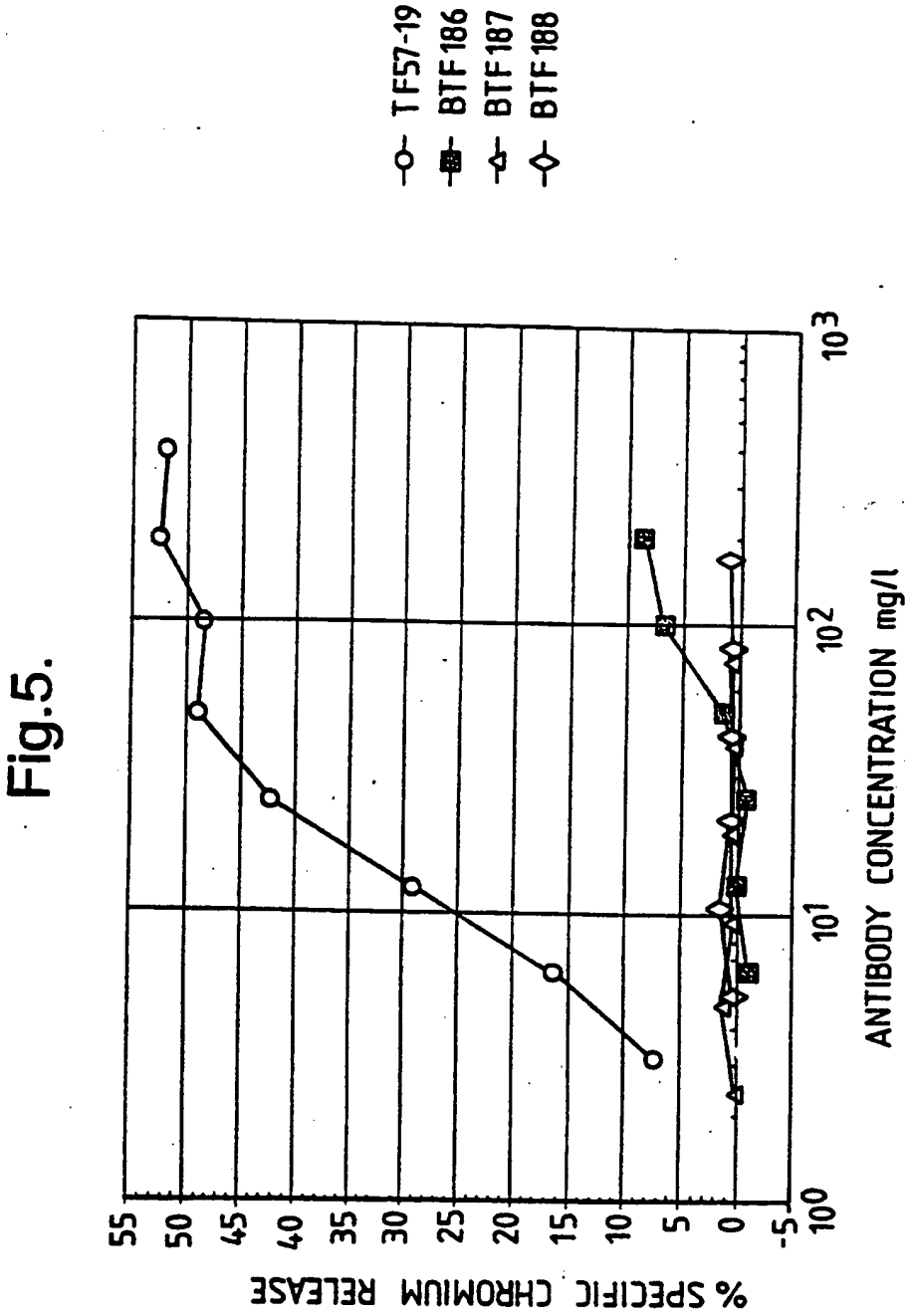
TF57-19
BTF186
BTF187
BTF188
% SPECIFIC CHROMIUM RELEASE
55
50
45
40
35
30
25
20
15
10
5
0
-5
ANTIBODY CONCENTRATION mg/l
10^0
10^1
10^2
10^3

Fig.5.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 94/01790

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/62 C07K19/00 C07K14/54 C07K14/55 C07K14/705
A61K39/395 C07K16/46

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,92 16562 (LYNXVALE LTD, GB) 1 October 1992 cited in the application	1-4,8, 11-23, 25-28, 32,35-38
Y	see the whole document	5,6,9, 10,29,30

Y	WO,A,92 08495 (ABBOTT BIOTECH, INC, US) 29 May 1992 see page 11, line 14 - page 20, line 1; examples 2,3	5,6,29, 30

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 December 1994	22. 12. 94
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	**Authorized officer** Nauche, S

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF CELLULAR BIOCHEMISTRY, vol.17B, February 1993 page 73 BIRKELAND, M. ET AL.; 'Cloning and expression of mouse OX-40 the homologue of a rat cell surface protein restricited to CD4-posisitve T blasts' & Keystone symposisum on cytokines and cytokine receptors : from cloning to the clinic, Keystone; Colorado; USA; 31 january- 7 frbruary 1993 Abstract E204 ---	9,10
X	INTERNATIONAL REVIEW OF IMMUNOLOGY, vol.10, no.2-3, 1993, USA pages 241 - 250 JOFFLIFFE, LINDA K.; 'Humanized antibodies : enhancing therapeutic utility through antibody' see the whole document ---	1-4
X	BIOTECHNIQUE, vol.4, no.3, 1986 pages 214 - 221 OI, V.T.; MORRISON S.L.; 'Chimeric Antibodies' see page 215, column 2, line 29 - page 215, column 3, line 23 ------	1-4

5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB94/01790

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Altough claim 38 is directed to a method of treatment of the human/ animal body as well as diagnostic methods, (Rule 39.1(iv)PCT) the search has been carried out and based on the alleged effects of the compound/ composition.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

...ormation on patent family members

Interna... ...l Application No
PCT/GB 94/01790

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9216562	01-10-92	EP-A-	0575407	29-12-93
WO-A-9208495	29-05-92	AU-A-	9059691	11-06-92
		CA-A-	2095836	10-05-92
		EP-A-	0574395	22-12-93